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(54) Title: ANTIGEN-LOADED CHITOSAN MICROPARTICLES FOR ORAL VACCINATION

(57) Abstract: The present invention relates to oral vaccines comprising chitosan microparticles loaded with antigenic material of an infectious agent causing respiratory disease in mammals or poultry for combating respiratory infectious disease. The invention also relates to pharmaceutical compositions comprising chitosan microparticles loaded with antigenic material of an infectious agent causing respiratory disease in mammals or poultry, for use in oral vaccines. Also, the invention relates to the use of such chitosan microparticles for the manufacturing of a vaccine for oral application for combating respiratory infectious disease, more preferably of a vaccine for oral application in maternally immune animals.

ANTIGEN-LOADED CHITOSAN MICROPARTICLES FOR ORAL VACCINATION

The present invention relates to oral vaccines for combating respiratory disease, that comprise chitosan microparticles, to pharmaceutical compositions comprising such particles and to the use of such particles for the manufacturing of vaccines for oral application.

Vaccination is one of the most frequently used methods for protecting animals against (the adverse effects of) infectious agents. Vaccination however, even when using efficient vaccines, is not always simple and straightforward.
First of all, most pathogens enter the body through the mucosa. And locally produced secretory IgA, produced as a reaction to the infection is considered to be amongst the most important protective local humoral immune factors. Therefore, vaccines should preferably be applied at the site of infection. However, conventional inactivated or subunit vaccines are often poorly immunogenic when given mucosally.
The main role in the production of IgA, constituting over 80% of all antibodies produced in mucosa associated tissues, is played by the various lymphoid tissues. There are several clearly defined groups of lymphoid tissues, such as the gut associated lymphoid tissue (GALT), the nasal associated lymphoid tissue (NALT) and the bronchus associated lymphoid tissue (BALT). Generally, antigens need to be applied topically to mucosal surfaces to elicit a good mucosal immune response. In practice, this means that for protection against e.g. infections of the enteric system, one would clearly prefer oral vaccination, with the aim of specifically triggering the GALT. For protection against respiratory infections, one would be highly motivated to apply a vaccine intranasally, in order to specifically stimulate the NALT.
Such intranasal application is however always complex and not always feasible if only from a point of view of animal handling. First of all, in order to ensure that a sufficient amount of vaccine is delivered into the nose, one has to avoid leakage/spillage during application and thereafter: the vaccine must remain on the spot. From a practical point of view, this is clearly not easy. Next to this, especially large animals such as horses, pigs and cows will not allow such applications easily. Moreover, the manufacturing process for dosage forms such as sprays for intranasal application is complex.

It is an objective of the present invention to first of all provide ways to circumvent the problems that accompany intranasal vaccination in this respect.

Surprisingly it was found now that protection against respiratory infectious disease can be obtained by oral vaccination with a vaccine that comprises chitosan microparticles loaded with antigenic material of an infectious agent causing respiratory disease in mammals or poultry. Such an oral route of vaccination using the said chitosan microparticles unexpectedly appeared to be well capable of inducing a sufficiently high immune response in the mucosa of the respiratory system to protect against respiratory infectious disease.

Therefore, a first embodiment of the present invention relates to a vaccine for combating respiratory infectious disease that has as its characteristics that it is an oral vaccine and that it comprises chitosan microparticles loaded with antigenic material of an infectious agent causing respiratory disease in mammals or poultry

Chitosan can be obtained through the deacetylation of chitin, the major compound of exoskeletons in crustaceans. Chitosan [α-(1→ 4)-2-amino-2-deoxy-β-D-glucan] is a mucopolysaccharide closely related to cellulose, which chemical properties are determined by the molecular weight, degree of deacetylation and viscosity. Chitosan has been reviewed in Paul, W. and Garside, C.P. (S.T.P.Pharma Sci. 10: 5-22, (2000)). Chitosan and its derivatives have been described more specifically in relation with the present invention by van der Lubben, I.M. et al, in Eur. Journ. Pharm. Sci. 14: 201-207 (2001).
The present invention relates i.a. to chitosan microparticles loaded with antigenic material. The loading of chitosan microparticles is described below, and is also described by van der Lubben, I.M. et al, in Biomaterials 22: 687-694 (2001).
Chitosan microparticles are known to target to the Peyer's patches of the gut associated lymphoid tissue (GALT). In poultry, the role of the Peyer's patches is fulfilled by the coecal tonsils and the GALT.
Chitosan has occasionally been tested for mucosal application, but, in line with the present knowledge of lymphoid tissues, applied directly to the mucosal surface to be protected. Merely as an example, intranasal application of chitosan has been tested in order to obtain IgA in the nasopharyngeal mucosa against influenza virus by Bacon A., et al. (Inf. & Imm. 68:5764-5770 (2000)). Intranasal application is, as mentioned above, however always complex from a point of nasal vaccine formulation and not always feasible if only from a point of view of animal handling.

A vaccine for oral application, also called an oral vaccine, is a vaccine that is specifically designed for this route of application and not or significantly less suitable for other routes of application.

Such a vaccine can have several forms. Basically, such a vaccine comprises chitosan microparticles loaded with antigenic material of an infectious agent causing respiratory disease in mammals or poultry and a carrier that makes it specifically suitable for oral application.

For inactivated vaccines, the amount of antigen administered is relatively critical compared to live attenuated vaccines. Therefore, inactivated (subunit-) vaccines must be administered efficiently, without much spillage.

The carrier can i.a. be an orally acceptable gel, for instance a so-called hydrogel. Such gels can be used for application of vaccines into the mouth of the animal to be vaccinated. They have the advantage that, due to their sticky character, they do not leak away. This ensures that the animal receives the necessary amount of antigen. Such gels, often based upon methyl-cellulose or alginates are known to man skilled in the art, and have been described i.a. in H.P. Fiedler, Lexicon der Hilfstoffe, Editio Cantor Verlag, Aulendorf, Germany.

Pills are also very efficient carriers suitable for oral administration, because the amount of antigen to be delivered can be very precisely defined. Capsules are comparably efficient. Numerous ways of making pills and using capsules are known in the art. Standard gelatin capsules are e.g. obtainable from Capsugel (Colmar, France).

The only disadvantage of pills, gels and capsules is that they have to be administered by physically handling the animal. This may or may not be easy, depending on the animal to be vaccinated and the number of animals to be handled. For a single administration to e.g. a horse or pet animal, they will be efficient and convenient. For the vaccination of e.g. large amounts of pigs, poultry or cows however, other applications are needed. In such cases, chitosan microparticles can e.g. be coated to food. This can be done very easily by spraying a suspension comprising a known amount of the chitosan microparticles according to the invention directly to a known amount of dry food pellets. Administration of a known dose of food will thus ensure the administration of a sufficient amount of vaccine.

Another attractive and frequently used way of vaccination is administration through the drinking water. Simple administration of a known amount of chitosan microparticles according to the invention to relatively small but known amounts of drinking water ensures correct vaccination doses. Animals that have been deprived

of water for a certain time will then ingest a sufficient amount of vaccine through the drinking water.
All these methods are by nature only suitable for oral application. They are not suitable for systemic application and they are not suitable for intranasal vaccination. Merely as an example: spraying chitosan microparticles in water into the nose would only be suitable on a laboratory scale, because most of the vaccine will be sneezed out or leaking out. Specific vaccines for intranasal application therefore use oily carriers and/or carriers comprising a surfactant: such additives are not necessary, and even not preferred for oral vaccines according to the invention. The other applications mentioned above are clearly not suitable for other than oral applications anyway.

Another embodiment of the invention relates to pharmaceutical compositions comprising chitosan microparticles loaded with antigenic material of an infectious agent causing respiratory disease in mammals or poultry, for use in oral vaccines. Such pharmaceutical compositions are considered to comprise the gels, pills, capsules or the water, and the antigen-loaded chitosan microparticles.

Still another embodiment of the present invention relates to the use of chitosan microparticles loaded with antigenic material of an infectious agent causing respiratory disease in mammals or poultry for the manufacturing of a vaccine for oral application for combating respiratory infectious disease.
Such manufacturing may e.g. comprise the admixing of the loaded chitosan microparticles and water, a gel, compounds for pills, or the filling of a capsule with loaded chitosan microparticles.

In all cases mentioned above the microparticles may or may not be coated with a coating that protects the microparticles until they leave the stomach. Such coatings are well-known in the art as enteric coatings. Eudragit, more specifically Eudragit L100, obtainable through Röhm (Darmstadt, Germany) is a well-known example of such a coating. These and comparable coatings are resistant to the acidic environment of the stomach, so pills or capsules coated with this kind of coating will only fall apart and set free their contents in the intestines. This kind of coatings can be used for the coating of pills and capsules, but it is equally possible to coat the microparticles as such. Such coatings can e.g. be applied to the chitosan

microparticles while they are in suspension, or after they have been freeze-dried and/or granulated.

Thus the coating can equally well be applied to the loaded chitosan microparticles according to the invention as such, before they are added to the drinking water, sprayed to food pellets or added to a gel. For oral vaccines, protection of the antigen with an enteric coating is considered by those skilled in the art to be highly desired, if not a necessity.

It was surprisingly found now, that the non-enteric-coated microparticles or capsules give even better vaccination results when compared with the enteric-coated capsules or microparticles. This is surprising because it would be expected that antigens, when entrapped in enteric-coated chitosan microparticles are even more resistant to the digestive processes of the stomach.

Thus, in a preferred form of this embodiment the vaccine according to the invention comprises chitosan microparticles that are not protected by an enteric coating.

The antigens (= antigenic material) to be used for loading the microparticles can be highly divers. Whole bacteria, parasites and virus particles can be used, provided that the size of the microparticles including the antigen (the bacterium or parasite) does not exceed 10 µm in diameter (for viruses this will usually be the case).

Preferably isolated antigens of these bacteria, parasites or viruses are used.

The following pathogens are well-known examples of respiratory diseases in poultry: *Haemophilus paragallinarum, Mycoplasma synoviae, Mycoplasma gallisepticum, Escherichia coli, Bordetella avium, Ornithobacterium rhinotracheale*, infectious laryngotracheitis virus, turkey rhinotracheitis virus, infectious bronchitis virus, *Pasteurella multocida* and *Riemerella anatipestifer.*

Well-known dog respiratory pathogens are e.g.: *Bordetella bronchiseptica*, Distemper virus, parainfluenza virus, *Mycobacterium tuberculosis, Mycobacterium bovis, Streptococcus pyogenes* and *Nocardia* sp.

Well-known cat respiratory pathogens are e.g.: *Bordetella bronchiseptica, Streptococcus pyogenes*, feline Herpesvirus I, *Chlamydia* and feline calicivirus.

Examples of horse respiratory pathogens are: equine influenza virus, equine Herpesvirus 4, *Streptococcus equi, Streptococcus zooepidemicus* and *Mycoplasma sp.*

Pig respiratory pathogens are e.g.: *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, swine influenza, Aujeszky's disease virus, *Bordetella bronchiseptica* and *Pasteurella multocida*.

As examples of cattle respiratory pathogens may serve: *Pasteurella haemolytica, Pasteurella multocida, Haemophilus somnus*, parainfluenzavirus III, bovine respiratory syncytial virus, bovine herpesvirus I, bovine viral diarrhea virus and adenovirus III.

The skilled person would know which antigens to select for the loading of the microparticles according to the invention.

Another notorious and universal problem in vaccination is the problem of maternal immunity. Maternal immunity is the kind of immunity found in very young animals, as a defense against infection in the first few weeks of their live. Animals do not have any significant immunity of their own at birth. Their immune system only starts developing after some time. Therefore, in order to survive the most vulnerable period newborn animals receive antibodies against those diseases "seen" by their mother through the colostrum. These antibodies are taken up through the intestinal wall in the first few days after birth. In poultry, the transfer of maternally derived antibodies takes place through the egg yolk. The maternal antibodies, which are mainly of the IgG type have a life span of about 6-10 weeks in mammals and about 3-4 weeks in poultry. This means that mammals and poultry are protected during the first 6-10, respectively 3-4 weeks of their live against most infectious pathogens. From a biological point of view, this is of course highly advantageous. From a point of view of vaccination however, the presence of maternally derived antibodies poses serious problems. If a newborn animal is vaccinated in the presence of maternally derived antibodies, the antigens present in the vaccine will be captured by the maternally derived antibodies. Therefore, they will no longer be able to trigger the animal's slowly developing immune system, so they will have no effect whatever. Even worse, the antigens will absorb (part of) the maternally derived antibodies and therefore the animal's defense is diminished.

As mentioned above, this problem of the existence of maternal antibodies in newborn animals is a well-known problem in vaccinology. For attenuated live vaccines a solution, although far from elegant may be the use of only slightly attenuated live strains. This has as a clear disadvantage that the animals will suffer significantly from the vaccination. For inactivated vaccines and subunit vaccines, there is no solution to the problem anyway. As a consequence of this problem, subunit vaccines can only

be given after the period in which maternally derived antibodies are present. Since immunity takes a few weeks to build up after vaccination, there clearly is a period between the loss of maternally derived antibodies and the building of protection after vaccination, during which the animal is not protected.

Even more surprisingly it was found now, that vaccines according to the present invention are not at all hampered by the presence of maternally derived antibodies. They are, through their novel way of application, capable of inducing an early immune response in spite of the presence of significant amounts of maternally derived IgG antibodies.

Therefore, a more preferred form of the present invention relates to the use of chitosan microparticles loaded with antigenic material of an infectious agent causing respiratory disease in mammals or poultry for the manufacturing of a vaccine for oral application for combating respiratory infectious disease in maternally immune animals.

Example 1

Animals and materials

Chitosan was obtained from Primex (Avaldsness, Norway). The viscosity of the chitosan used was measured as 1% (w/v) chitosan in 1% (v/v) acetic acid in MilliQ water on a rotation viscosimeter (Haake, Karlsruhe, Germany) and found to be 13 mPas.

The degree of deacetylation, as given by the supplier, was 93%. Tween 80® was purchased from Sigma (Bornem, Belgium).

ApxI, ApxII, ApxIII and OMP antigens were obtained as described in European Patent EP 0 453 024 of AKZO Nobel N.V. Eudragit L100 was obtained from Röhm (Darmstadt, Germany). Gelatin capsules (size 1) were obtained from Capsugel (Colmar, France).

Conventional female and male pigs (27 in total) were used, being 6 weeks old at the start of the experiment.

Chitosan microparticles

Chitosan microparticles were prepared and characterized as described before (Van der Lubben, I.M., J.C.Verhoef, A.Van Aelst, G.Borchard and H.E.Junginger. Chitosan microparticles for oral vaccination : preparation, characterization and preliminary in vivo uptake studies in murine Peyer's patches. *Biomaterials* **22**:687-694 (2001)

Briefly, a chitosan solution of 0.25% (w/v) was prepared in MilliQ water containing 2% (v/v) acetic acid and 1% (w/v) Tween® 80. Then, 2.5 ml of 10% (w/v) sodium sulfate in water was added to 250 ml chitosan solution under stirring and sonication. The microparticle suspension was subsequently centrifuged for 25 min (2750 rpm). The pellet was resuspended in MilliQ water to wash the microparticles and centrifuged again. After repeating this washing step, the pellet was freeze-dried overnight using a Christ freeze dryer (Christ, Osterode am Harz, Germany) connected to a pump (Leybold, Karlsruhe, Germany). The size of these chitosan microparticles was found to be 4.3 ± 0.7 (mean ± SD) μm and were positively charged (20 ± 1 mV).

Association of Apx toxoids and OMP to chitosan microparticles

To determine if all three Apx toxoids and OMP could be associated efficiently to chitosan microparticles, both loading and release studies were carried out.

The antigen loading of microparticles was performed by incubating 1% (w/v) chitosan microparticles and 500 Units per ml of each antigen in Tris (hydroxymethyl)aminomethane) buffer (0.04 M; pH 7.4) under mild shaking at 4°C.

After overnight incubation, the suspension was centrifuged (14.000 rpm for 3 min) and the antigen content in the supernatant was determined.
The release of the loaded antigens was determined by resuspending the loaded microparticles in phosphate buffered saline (PBS; pH 7.4) and incubating them at 37°C for 3.5 hrs. Then the microparticle suspension was centrifuged (14.000 rpm for 30 sec) and the amount of released antigen was determined in the supernatant.
Where units are used, the relation between units and micrograms of antigen are as follows: ApxI: 1 U = ca. 0.5 - 1 µg eiwit, ApxII: 1 U = ca. 4 - 8 µg eiwit, ApxIII: 1 U = ca. 0.5 - 1 µg eiwit, OMP: 1 U = ca. 1 µg eiwit.
In the first association experiment, the loading and release were determined, using the Lowry protein assay for the analysis of the antigens. In this experiment two different antigen mixtures were loaded to the chitosan microparticles: one mixture of Apx I, II and III and the other of Apx I, II, III and OMP. After analyzing the loading efficacy, the release was determined. The loading efficacy was calculated as follows: LE= [(total amount antigens)-(free antigens)]/total antigens.

In the second experiment, the loading with Apx I, II, III and OMP was performed as described above, but now the total amounts and amounts of free antigens were measured with specific Apx I, II and III enzyme-linked immunosorbent assays and gel scanning of the OMP antigen (see below).

Specific enzyme-linked immunosorbent assays (ELISA's) for ApxI, ApxII and ApxIII antigen and gel scanning for OMP antigen

Enzyme-linked immunosorbent assays (ELISA's) were used to quantify the antigenic mass of the Ap antigens, ApxI, ApxII and ApxIII toxoids in the respective antigens. In short, the tests were done as follows. The wells of a polystyrene microtitre plate were coated with specific monoclonal antibodies (against either ApxI, ApxII or ApxIII). Serial two-fold dilutions of antigen were incubated in the wells. Antigen captured by the adsorbed antibody was quantified by: i) a detecting monoclonal antibody which had the same specificity as the adsorbed antibody, but which was peroxidase-labeled; ii) TMB as substrate; iii) measuring the optical density at 450 nm. The antigenic mass was determined relative to a standard batch of each antigen and expressed in Units/ml.
The antigenic mass of the Ap 42kD-OMP antigen was determined using SDS-PAGE and gel scanning. In short, this method is as follows. Various dilutions of the OMP test antigen were run together with a standard batch of OMP antigen, and stained

with Coomassie Brilliant Blue. The gel was scanned, and by comparing the area of the 42kD band in the test samples with the area of the 42kD band of the standard batch the amount of OMP in the test samples was calculated, and expressed in Units/ml.

Vaccination and challenge study in pigs

Three groups of 6 pigs each were orally vaccinated with the following formulations:
i) group 1: Apx I, II, III and OMP in Tris buffer (pH 7.2), without chitosan microparticles, given in an enteric-coated gelatin capsule (negative (= chitosan-free) control). Such a capsule is protected until it leaves the stomach;
ii) group 2: Apx I, II, III and OMP simultaneously associated to chitosan microparticles, given in a non-coated gelatin capsule;
iii) group 3: Apx I, II, III and OMP simultaneously associated to chitosan microparticles, given in an enteric-coated gelatin capsule.
In addition, 4 non-vaccinated pigs were used as placebo controls in the challenge studies (group 4). In Table 1 the experimental setup of the vaccination study is shown. The pigs from groups 1-3 were primed on three consecutive days in week 1 and boosted on three consecutive days in week 4. Each pig received in total 400 Units of each antigen during priming vaccination and another 400 Units during boosting.
Prior to the first vaccination, one week after boosting and 48 hours after challenge (see below), blood samples of about 10 ml were collected from the jugular vein in a vacutainer system after anaesthetizing the pigs with halothane and N_2O. Blood samples were stored overnight at ambient temperature and then centrifuged (3750 rpm for 20 min) to obtain serum. Serum samples were stored at -20°C until they were used to determine the titers of serum IgG antibodies specifically directed against Apx I, II and III and OMP.

In order to perform the challenge, all pigs were anesthetized by inhalation of halothane and N_2O. Then $5*10^3$ colony forming Units of Actinobacillus pleuropneumoniae (Ap) serotype 9, ref strain 13261 in a volume of 10 ml were inoculated endobronchially. During the following 48 hrs, the physical condition, rectal temperature and clinical signs were recorded at 3, 6, 9, 12, 15, 24, 30, 36, 42 and 48 hours. If pigs developed severe clinical disease, for example dyspnoea or evident depression, they were euthanized by an overdose pentobarbital.

After 48 hours, the remaining pigs were also sacrificed with an overdose of pentobarbital. The lungs were dissected and evaluated for Ap-specific lung lesions. After counting of the lung lesions, five individual lung lobes were given scores from 1 to 5 (i.e. from 1 being not infected, to 5 being completely covered with lesions). The total scores per pig were added up according to an adapted method of Hannan et al. *Res. Vet. Sci 33:* 76-88 (1982).

Antibody ELISA's (Apx I, II and III and OMP)

Antibody ELISA's were used to determine the titers of serum IgG antibodies in pig sera specifically directed against ApxI, ApxII, ApxIII and 42kD-OMP from Ap. In short, these tests were done as follows. The wells of a microtitre plate were coated with either purified ApxI or ApxII or ApxIII or OMP with a standardized concentration. Serial two-fold dilutions of test sera were incubated in the wells. The bound antibodies were quantified using goat-anti-swine IgG peroxidase conjugate and TMB substrate. The optical density was measured at 450 nm. The mean background value measured in the negative reference serum was used for calculation of the antibody titers of the test sera. A titer of > 1:128 or a $\log_2$ [reciprocal titer] of > 7 was considered as positive.

RESULTS

Association of Apx toxins and OMP to chitosan microparticles

In order to investigate whether OMP and ApxI, II and III could be associated efficiently to chitosan microparticles, loading and release studies were carried out. In the first association experiment, two antigen mixtures were tested for their association to chitosan microparticles: Apx I, II and III, without and with OMP. Since Apx I, II and III are structurally and biochemically very similar, it was assumed that these antigens would demonstrate similar association behavior. After loading, the microparticles were incubated in PBS at 37°C to determine the antigen release. Both the loading and release were determined by means of an non-specific protein assay. From these experiments it was concluded that about 90% of both antigen mixtures associate to the chitosan microparticles. Subsequent release studies showed that in case of Apx toxoids and a mixture of Apx toxoids and OMP, only about 8 +/- 6 % of the total loaded antigen was released in 3.5 hrs (Table 2).

To investigate whether all separate antigens were able to associate to the chitosan microparticles, loading and release experiments for each individual antigen were

performed. None of the antigens Apx I, II, III and OMP could be detected in the supernatant after loading, indicating that at least 80% of each antigen was associated to the microparticles. Moreover, the concentration of each antigen found in the release experiment was below the detection limit (<30%; Table 2)
Thus the present association experiments demonstrate that Apx I, II, III and OMP can be efficiently associated to chitosan microparticles if they are loaded simultaneously. It is not expected that one of the antigens associates less efficiently, since the separate antigen association experiments clearly showed that all antigens could be equally well entrapped within the microparticles.

Vaccination and challenge study: survival and lung lesions

Three groups of pigs were orally vaccinated and boosted with either antigens in buffer in an enteric-coated gelatin capsule (negative control) or antigen-loaded chitosan microparticles in a non-coated gelatin capsule, or antigen-loaded chitosan microparticles in an enteric-coated gelatin capsule. After endobronchial inoculation with *A. pleuropneumoniae* (Ap) the pigs were monitored during 48 hrs. Table 3 gives an overview of the survival rates of the challenged animals. Pigs either died spontaneously or were euthanized if they developed severe clinical disease. All non-vaccinated pigs (group 4) died or were euthanized within 36 hours, indicating that the inoculated bacteria caused severe pneumonia within 48 hrs and, thus, were highly virulent. At the end of the monitoring period, all pigs orally vaccinated with antigen-loaded chitosan microparticles in a gelatin capsule (group 2) were still alive, while only one animal in the negative control (group 1) survived. Of the pigs receiving antigen-loaded chitosan microparticles in an enteric-coated capsule (group 3), still half of the group survived, in spite of the severe challenge.
The physical condition of the animals during the first 48 hours after the inoculation was also monitored. Pigs vaccinated with antigen-loaded chitosan microparticles in a gelatin capsule did not show any obvious signs of pneumonia during the challenge study. They behaved normal and kept eating. In the group with non-vaccinated pigs, about 75% of the animals were coughing and showed dyspnoea and depression within 12 hours after inoculation. In the pigs that would eventually die in the group of negative controls (group 1) and in the group vaccinated with antigen-loaded chitosan microparticles in enteric-coated capsules (group 3), the onset of pneumonia was firstly observed after 18 to 24 hours.
Directly after the pigs died or were euthanized, the lungs were examined for lesions. As becomes clear from Table 4, only in pigs vaccinated with antigens associated to

chitosan microparticles in gelatin capsules, the lungs showed significantly less lesions than the lungs of pigs in the negative controls (group 1). In the other vaccinated group, group 3, the lung lesions were not significantly different from those observed in group 1 or 4.

Systemic immune responses

In all vaccinated and non-treated pigs, blood samples to determine the systemic immune response were taken before and after vaccination and after challenge. IgG titres were separately analyzed for OMP, Apx I, Apx II and Apx III specific antibodies. As follows from figures 1-4, prior to the first vaccination, significant levels of maternally derived IgG antibodies against Apx I, II and III and OMP were found to be present in the serum of pigs in all groups. In particular the ApxII antibody titers were relatively high. In none of the treatment groups any seroconversion regarding any of the four antibodies was demonstrated.

Conclusion

In the present study, it was found that Apx toxoids and OMP could be efficiently and simultaneously associated to chitosan microparticles. A subsequent vaccination-challenge study demonstrated that pigs orally vaccinated with antigen-loaded chitosan microparticles in non-coated gelatin capsules were fully protected against Ap infection induced by endobronchial inoculation. During the 48-hour post-challenge monitoring period, no signs of Ap-related pneumonia or evident symptoms of systemic disease were found in this group. Macroscopical examination of the lungs at the end of the challenge study revealed that the protected animals had significantly fewer lesions than non-vaccinated pigs and animals orally vaccinated with the antigens without microparticles.

Oral administration of Apx toxoids and OMP associated to chitosan microparticles did not induce a detectable systemic humoral immune response against any of these antigens, since no seroconversion after two vaccinations was measured in any of the vaccinated groups. Nevertheless, chitosan-associated Ap antigens induced partial or even complete protection against a virulent App challenge.

The extremely severe challenge in an artificial challenge model such as endobronchial inoculation with *Actinobacillus pleuropneumoniae* after vaccination and boosting clearly demonstrated that pigs vaccinated with chitosan microparticles in a gelatin capsule were fully protected and that they all survived a heavy challenge.

It can therefore be concluded that chitosan microparticles are very efficient in the oral delivery of multiple antigens for protection against respiratory infections.

Moreover, this result was obtained in the presence of significant titres of maternally derived IgG against all four Ap-antigens. This shows that vaccines according to the present invention provide very efficient protection in maternally immune animals.

The anatomy of the gastro-intestinal tract of pigs is known to be similar to that of human beings. The present results obtained in pigs therefore indicate that chitosan microparticles as oral vaccine carrier systems are also suitable for human purposes. Especially in the third world, the avoidance of injection needles, trained personnel, cool and sterile storage and transport may substantially increase the efficiency of vaccination campaigns.

Table 1

Experimental setup of the vaccination and challenge study.

Group	Formulation	N	Route	Chitosan Microp. (per week) (mg)	Antigens (per week) (Units)	Vaccination
1	Apx I, II, III and OMP in enteric-coated gelatin capsule	6	Oral	-	400	Weeks 1 & 4 3 cons. days
2	Apx I, II, III and OMP/Chitosan microparticles in non-coated gelatin capsule	5	Oral	22	400	Weeks 1 & 4 3 cons. days
3	Apx I, II, III and OMP/Chitosan microparticles in enteric-coated gelatin capsule	6	Oral	22	400	Weeks 1 & 4 3 cons. days
4	Not applicable (NA)	4	NA	NA	NA	None

N, number of pigs

Table 2

OMP, Apx I, II and III loading efficacy and release as determined by both specific antigen ELISA's and a nonspecific protein assay.

Antigen(s)	Loading Efficacy (%)	Release (%)
Protein assay		
Apx I, II and III	89 ± 2	8 ± 6
Apx I, II and III + OMP	89 ± 2	8 ± 6
Antigen ELISA and immunoblot		
Apx I	> 80	< 30
ApxII	> 80	< 30
ApxIII	> 80	< 30
OMP	> 80	< 30

Values are given as mean ± SD

Table 3

Number of pigs surviving the challenge for more than 12, 24, 36 and 48 hrs after inoculation (percentage of survivors per group and survivors/total group). Pigs were orally vaccinated with Apx toxoids and OMP in a gelatin capsule (neg. control), Apx toxoids and OMP associated to chitosan microparticles in a gelatin capsule and Apx toxoids and OMP associated to chitosan microparticles in an Eudragit coated capsule.

Group	Treatment	Time (hours) 12	24	36	48
1	App antigens without microparticles	100% (6/6)	83% (5/6)	67% (4/6)	17% (1/6)
2	Loaded microparticles in gelatin capsule	100% (5/5)	100% (5/5)	100% (5/5)	100% (5/5)
3	Loaded microparticles in coated capsule	100% (6/6)	83% (5/6)	67% (4/6)	50% (3/6)
4	None	100% (4/4)	75% (3/4)	0% (0/4)	0% (0/4)

Table 4

Total lung score determined by summarising the score of the 5 lung lobes of each group. Each lobe was given a score between 1 (no alterations) and 5 (total lung lobe covered with lesions). Data are expressed as mean ± SD. Pigs were orally vaccinated with Apx toxoids and OMP in a gelatin capsule (neg. control), Apx toxoids and OMP associated to chitosan microparticles in a gelatin capsule and Apx toxoids and OMP associated to chitosan microparticles in an Eudragit coated capsule. Student's t-test ($p < 0.05$)

Group	Treatment	Lung scores
1	App antigens without microparticles	16 ± 4
2	Loaded microparticles in gelatin capsule	8 ± 3
3	Loaded microparticles in coated capsule	12 ± 6
4	None	16 ± 1

Legends to the figures

Figure 1 OMP (a), ApxI (b), Apx II (c) and Apx III (d) specific IgG titres determined before vaccination (black columns), post boosting (white columns) and after challenge (grey columns). Pigs were orally vaccinated with Apx toxoids and OMP in a gelatine capsule (antigens without chitosan microparticles (mp's)), Apx toxoids and OMP associated to chitosan microparticles in a gelatin capsule (loaded mp's (gelatin)) and Apx toxoids and OMP associated to chitosan microparticles in an Eudragit coated capsule (loaded mp's (coated)).

Claims

1) Vaccine for combating respiratory infectious disease, characterized in that said vaccine is an oral vaccine, and said vaccine comprises chitosan microparticles loaded with antigenic material of an infectious agent causing respiratory disease in mammals or poultry.
2) Vaccine according to claim 1, characterized in that the chitosan microparticles are not protected by an enteric coating.
3) Pharmaceutical composition comprising chitosan microparticles loaded with antigenic material of an infectious agent causing respiratory disease in mammals or poultry, for use in an oral vaccine.
4) Pharmaceutical composition according to claim 3, characterized in that said composition comprises chitosan microparticles that are not protected by an enteric coating.
5) Use of chitosan microparticles loaded with antigenic material of an infectious agent causing respiratory disease in mammals or poultry for the manufacturing of a vaccine for oral application for combating respiratory infectious disease.
6) Use of chitosan microparticles loaded with antigenic material of an infectious agent causing respiratory disease in mammals or poultry for the manufacturing of a vaccine for oral application for combating respiratory infectious disease in maternally immune animals.
7) Use according to claim 5 or 6, characterized in that the used chitosan microparticles are not protected by an enteric coating.

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Figure 1a

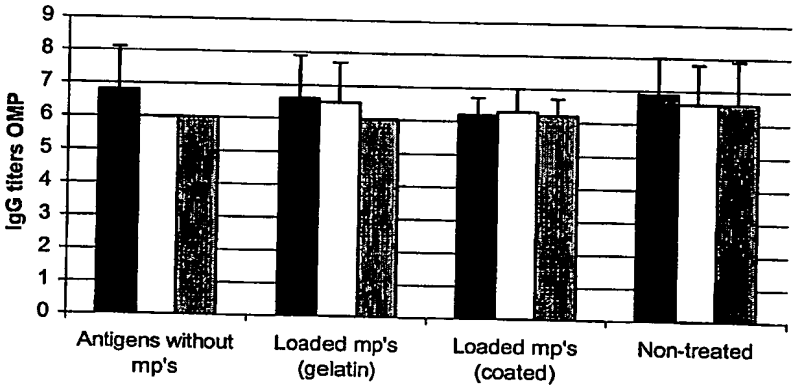

Figure 1b

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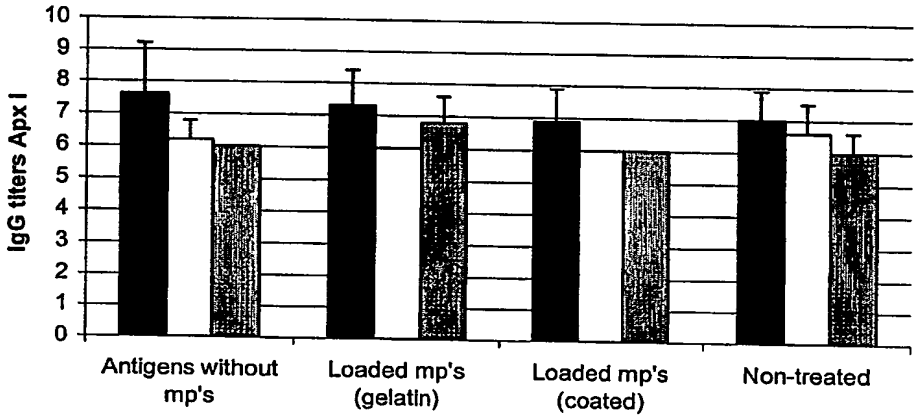

Figure 1c

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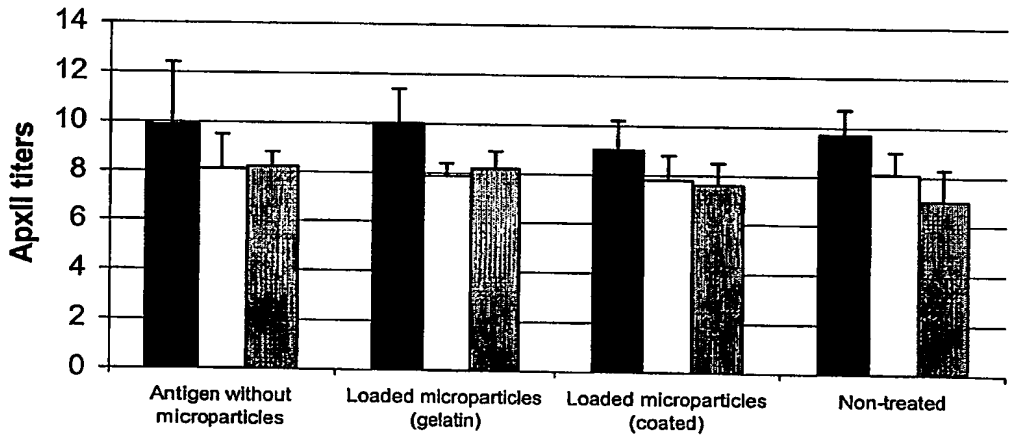

Figure 1d

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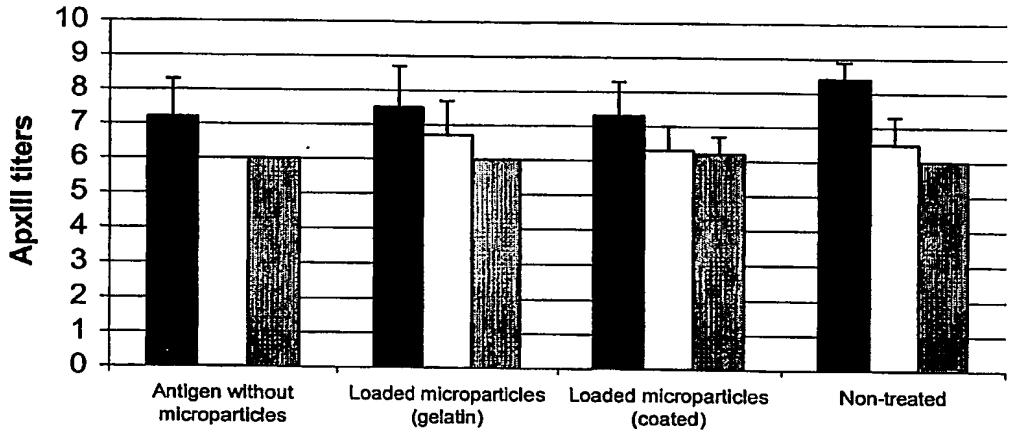

INTERNATIONAL SEARCH REPORT

PCT/EP 03/03875

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/385 A61K39/00 A61K39/02 A61K9/16 A61K47/36
A61P11/00 A61P31/04 A61P37/00 //A61K39/102

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 June 2003	24/07/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lechner, O

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	SENEL S ET AL: "Drug permeation enhancement via buccal route: possibilities and limitations" JOURNAL OF CONTROLLED RELEASE, ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, NL, vol. 72, no. 1-3, 14 May 2001 (2001-05-14), pages 133-144, XP004246443 ISSN: 0168-3659 abstract page 141, left-hand column, paragraph 3 -right-hand column -& SENEL S ET AL: "Enhancing effect of chitosan on peptide drug delivery across buccal mucosa" BIOMATERIALS, ELSEVIER SCIENCE PUBLISHERS BV., BARKING, GB, vol. 21, no. 20, October 2000 (2000-10), pages 2067-2071, XP004228763 ISSN: 0142-9612 --- -/--	1-7

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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